

AMENDMENT

CLAIMS

1.- A method of obtaining new astaxanthin overproducing strains of *Xanthophyllomyces dendrorhous* (*X. dendrorhous*) consisting in inducing mutation of a parent strain of *X. dendrorhous* by incubating said parent strain under mutagenic conditions and by selecting the mutants obtained thereof, characterised because a first selection of astaxanthin overproducing mutants is achieved by growing 10 the mutants on solid medium containing either inhibitors of the synthesis of steroids or compounds that alter the redox potential of the cell and then astaxanthin overproducing mutant strains were selected subsequently as a function of their yield on solid medium measured as:

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- i. deeper red coloration than the parent strain
- ii. production of astaxanthin in darkness
- iii. production of astaxanthin at temperatures over 20°C
- iv. production of astaxanthin using sucrose as carbon source.

20 2.- A method according to claim 1 characterised by inducing mutation of the parent strain by incubating said strain in an appropriate culture medium containing a mutagenic agent selected among ethylmethanesulfonate (EMS) and N-methyl-N'-nitrosoguanidine (NTG) or irradiating said culture medium containing the parent strain of *X. dendrorhous* with ultraviolet arrays (UVA).

30 3.- A method according to any of the claims 1 or 2 in which the parent strain of *X. dendrorhous* was VKPM Y-2476.

4.- A method according to any of the claims 1-3 characterised by selecting the mutants by growing them in solid medium containing as inhibitor of the steroid synthesis a compound selected among  $\beta$ -ionone, imidazole,

diethylamine, 2-methylimidazole, nystatin and diphenylamine or, as compound that alter the redox potential of the cell, a compound selected among duroquinone or hydrogen peroxide.

5 5.- A method according to any of the claims 1-4 characterised by selecting the mutants by growing them in solid medium at 24°C.

10 6.- Astaxanthin overproducing mutants obtainable by the method of claims 1-5 characterised by possessing extrachromosomal elements consisting in linear double strand DNA plasmids and capable of producing at least 4000 ppm of astaxanthin after 6-7 days by flask fermentation.

7.- Astaxanthin overproducing mutants according to claim 6 characterised by producing at least 5000 ppm of astaxanthin after 7-9 days in industrial fermentation.

15 8.- Process for producing astaxanthin characterised in culturing in a suitable medium at appropriate growth conditions the mutants of claims 6 or 7 or derivatives thereof having the same extrachromosomal elements and having the same level of astaxanthin production.

20 9.- A process of fermentation according to claim 8, characterized in that duroquinone is added during the fermentation process.

25 10.- A process of fermentation according to claim 9, characterized in that duroquinone is added at a concentration of 25-50  $\mu$ M.

11.- A process of fermentation according to claim 8, characterized in that retinal is added during the fermentation process.

30 12.- A process of fermentation according to claim 11, characterized in that retinal is added at a concentration of 35  $\mu$ M.

13.- A process of fermentation according to claim 8, characterized in that trisporic acids are added during the fermentation process.

14.- A process of fermentation according to claim 13, characterized in that the trisporic acids are added at a concentration of 50-100 µg/ml.

5 15.- A process of fermentation according to claim 8, characterized in that glutamate is added during the fermentation process.

16.- A process of fermentation according to claim 15, characterized in that glutamate is added at a concentration of 5.5 mg/ml.

10 17.- A process of fermentation according to claim 8, characterized in that medium 5 described in Table I of the description is used for the fermentation process.

15 18.- A process of fermentation according to any of the claims 8-17, characterized in that the fermentation medium is illuminated during the fermentation process.

19.- A process of fermentation according to claim 18, characterized in that the source of illumination used is white light.

20 20.- A process of fermentation according to claim 18, characterized in that the source of illumination used is ultraviolet light.

25 21.- A process of fermentation according to any of the claims 18-20, characterized in that illumination is carried out from the start to the end of fermentation, preferably from 40 to 200 hours.

22.- A process of fermentation according to claim 21, characterized in that cycles of 6 hours of illumination / darkness are used.

30 23.- A process of fermentation according to any of the claims 8-22, characterized in that:

(a) Inocula of *X. dendrorhous* are seeded.

(b) The inocula of *X. dendrorhous* are cultivated for 48 hours at 20°C.

(c) Phases of primary culture of *X. dendrorhous* are seeded with about 0.4% (v/v) of the inoculum phase.

(d) The primary phases of *X. dendrorhous* are cultivated for 48-54 hours at 17-20°C.

5 (e) Each fermenter is seeded with 20% (v/v) of the primary phases of *X. dendrorhous*.

(f) The fermentation is incubated at 18-20°C for 60-72 hours and then at 17°C for 5-7 days.

24.- Biomass of *X. dendrorhous* with nutrient and 10 pigmenting value, obtainable by the fermentation process described in claims 8 to 23, for use in food for humans and animals.

25.- Biomass according to claim 24, characterized in that it contains:

15 a) A concentration of at least 5000 µg/g of astaxanthin;  
b) A concentration of at least 7400 µg/g of total carotenoids;  
c) A concentration of at least 15% of proteins and  
d) A concentration of at least 15% of carbohydrates.

20 26.- Compounds for animal food that consist of or contain the biomass of claims 24 and 25.

27.- Compounds for human food that consist of or contain the biomass of claims 24 and 25.